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A COMPARISON OF SEVERAL SEROLOGIC TESTS TO DETECT ANTIBODIES TO *TOXOPLASMA GONDII* IN NATURALLY EXPOSED BOTTLENOSE DOLPHINS (*TURSIOPS TRUNCATUS*)

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ABSTRACT: *Toxoplasma gondii* infection in marine mammals is intriguing and indicative of contamination of the ocean environment and coastal waters with oocysts. In a previous study, 138 of 141 (97.8%) bottlenose dolphins (*Tursiops truncatus*) from the coasts of Florida and California had antibodies to *T. gondii* by the modified agglutination test (MAT). Although the MAT has been found to be highly sensitive and specific for *T. gondii* antibodies from several species of terrestrial animals, it has not yet been validated for *T. gondii* infections in marine mammals. Furthermore, *T. gondii* has yet not been isolated from dolphins. In the present study, sera from 146 (60 from the 2004 samples and 86 from the 2003 samples) *T. truncatus* from the coastal areas of South Carolina and Florida were tested for antibodies to *T. gondii*. Sera from 2004 were tested by the MAT, the indirect fluorescent antibody test (IFAT), the Sabin–Feldman dye test (DT), an indirect hemagglutination test (IHAT), an enzyme-linked immunosorbent assay (ELISA), and Western blot. All 60 dolphins were seropositive, with MAT titers of 1:20 in 3, 1:40 in 19, 1:80 in 29, 1:160 in 2, 1:1,280 in 3, 1:2,560 in 2, and 1:5,120 or higher in 2, and these results were confirmed in another laboratory. The DT titers of these dolphins were <1:10 in 53, 1:800 in 3, 1:1,600 in 2, and 1:3,200 in 2. The IHAT titers were <1:64 in 52, 1:128 in 1, 1:512 in 2, and 1:2,048 in 5. The IFAT titers were <1:20 in 3, 1:20 in 11, 1:40 in 36, 1:80 in 2, 1:160 in 1, and 1:320 or higher in 7. All 7 DT-positive dolphins had high MAT titers, but 2 were negative by the IHAT. Western blot results closely followed MAT results; ELISA results matched MAT results, which were 1:40 or higher. In sera from the 2003 samples, MAT antibodies were found in 86 of 86 dolphins with titers of 1:25 in 29, 1:50 in 23, 1:100 in 27, 1:200 in 3, 1:1,600 in 1, and 1:3,200 in 3; these sera were not tested by other means. Overall, MAT antibodies were found in all 146 dolphin sera tested. Because marine mammals are considered sentinel animals indicative of contamination of the coastal and marine waters by *T. gondii* oocysts, serologically positive infections need to be validated by the detection of *T. gondii* organisms in the tissues of seropositive animals.

Toxoplasma gondii infections have been reported in many homeothermic animals, including several species of marine mammals (Dubey and Beattie, 1988; Dubey et al., 2003). Recently, concerns have been raised that *T. gondii* may be a major cause of mortality in sea otters (Thomas and Cole, 1996; Lindsay et al., 2001; Miller, Gardner, Kreuder et al., 2002; Hanni et al., 2003; Kreuder et al., 2003). Viable *T. gondii* was isolated from 15 of 67 (Cole et al., 2000) and 24 of 75 (Miller, Gardner, Packham et al., 2002) dead sea otters, indicating that *T. gondii* infection is common in this animal. It has been suggested that sea otters become infected with *T. gondii* oocysts present in freshwater coastal runoff (Miller, Gardner, Kreuder et al., 2002). Reports of *T. gondii* infections in marine mammals were recently summarized (Dubey et al., 2003; Fayer et al., 2004).

Unlike sea otters, there are only a few reports of serologically confirmed toxoplasmosis in dolphins (reviewed in Dubey et al., 2003; Fayer et al., 2004). Antibodies to *T. gondii* were found in 91 of 94 and 47 of 47 of bottlenose dolphins (*Tursiops truncatus*) from the coasts of California and Florida, respectively (Dubey et al., 2003), using the modified agglutination test (MAT). These observations suggest either that the prevalence of *T. gondii* in these dolphins is indeed alarmingly high or that the MAT does not specifically detect *T. gondii* antibodies in dolphin sera. The objective of the present study was to evaluate antibodies to *T. gondii* in the sera of naturally exposed bottlenose dolphins by several serologic tests.

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MATERIALS AND METHODS

Naturally infected dolphins and sample collection

A multidisciplinary study initiated in 2003 evaluated the health of bottlenose dolphin populations at 2 U.S. sites in the southeastern United States, i.e., Charleston, South Carolina (CHS), and the Indian River Lagoon, Florida (IRL), to assess anthropogenic and environmental factors that might affect dolphin health. During the summer seasons of 2003 and 2004 (June in the IRL and August in CHS), 155 bottlenose dolphins were captured, examined, sampled, marked, and safely released. Dolphins were encircled using a 400- × 6-m seine net and then transferred to the deck of a boat for examination and sampling. A comprehensive, standardized protocol included physical and ultrasound examination, morphometric measurements, and the collection of blood, urine, feces, blubber, and skin tissue for hematology, serum chemistry, microbiology, immunology, contaminants, and a suite of biomarkers. Whole blood was collected from superficial fluke veins. Serum for serological testing was collected in vacutainers containing a clot activator and serum separator, and it was centrifuged on the boat. The 2004 serum samples were shipped overnight using cold packs, and the 2003 samples that were banked at −80 C were shipped frozen to the Animal Parasitic Diseases Laboratory (APDL), U.S. Department of Agriculture, Beltsville, Maryland. Sera collected from the 2003 sampling were tested after the results from the 2004 sampling were analyzed (Table I). Age was estimated by examination of postnatal dentine layers in an extracted tooth (Hohn et al., 1989). Ages were known for 51 of 60 and 68 of 86 dolphins during the 2004 and 2003 samplings, respectively, and more males than females were captured and sampled (35 and 55 for the 2004 and 2003 samplings, respectively).

Study sites

Indian River Lagoon, Florida: The IRL is a shallow-water ecosystem that accounts for 40% of Florida's central east coast. The IRL extends 250 km from the Ponce De Leon Inlet in the north to the Jupiter Inlet in the south and is composed of 3 estuarine bodies of water: the Indian River, the Banana River, and the Mosquito Lagoon. There is a limited exchange of waters between the lagoon and the ocean because of the small size of the inlets and the shallow nature of the lagoon. Limited circulation and tidal exchange increases the vulnerability of the lagoon to pollutants. Water quality in the IRL has deteriorated during the past several decades due to significant watershed alteration and land drainage patterns. The major cause of water quality decline is freshwater and stormwater discharges that alter water clarity and salinity as well as facilitate the introduction of increased nutrients and pollutants into the system (Scott et al., 2003). All of Florida's sugarcane, approximately 38% of the citrus, and 42% of the vegetable crops are grown in an area that drains into the IRL (Miles and Pfeuffer, 1997). Presently, there is concern for the overall health of the IRL ecosystem due to the destruction of the sea grass habitat, the alteration of water flow, and the decline in water quality. Dolphins in the IRL recently experienced an unusual mortality event (Marine Mammal Commission, 2002), and many have been diagnosed with a variety of skin lesions, including proliferative ulcerative dermatitis due to protozoans and fungi, dolphin pox dermatopathy, and a vesicular dermatopathy of unknown etiology (Bossart et al., 2003). There is a need to study the health status of populations that are depleted or show signs of epidemic disease and/or high mortality and that are located in areas where the habitat is being intensely altered or affected by human influences.

Charleston, South Carolina: Charleston Harbor is a 1,200-square mile estuarine environment formed by 3 river systems, i.e., the Cooper, the Ashley, and the Wando. It is located in the central portion of South Carolina, and the river systems form the third largest estuarine drainage area in South Carolina, with more than 26,000 ha of valuable marshland and tidal habitat (Tiner, 1977). The Charleston Harbor estuary has a soft mud bottom. Its average depth is 12 m at low tide and varies in width from 120 to 300 m. The Charleston Harbor–Cooper River coastal plain measures 66 km^2 with an additional 105 km^2 of intertidal wetlands. The estuary is subject to semidiurnal tides with a mean range of 1.6 m near the ocean (National Ocean Service, 1988). Long-term trends in water quality monitoring and recent scientific research suggest that waste load assimilation, nonpoint source runoff impacts, contaminated sediments, and toxic pollutants are key environmental issues for this area. In addition, several 'hot spots' showing high levels of heavy metals and organic compounds have been identified. High concentrations of anthropogenic trace metals, polychlorinated biphenyls, and pesticides have been found in the sediments of the Charleston Harbor and the Ashley and Cooper rivers (Long et al., 1998). The Charleston Harbor estuary is surrounded by urban development and has the second largest container port on the Atlantic seaboard; additionally, it has 2 sewage treatment plants that discharge into the basin. The Cooper River has the greatest number and density of industrial and port facilities, and more than 95% of the total pollutant loads to the Cooper River are believed to be from point sources.

In addition to the Charleston Harbor estuarine areas, the study area for the CHS dolphin health assessment study extended to an adjacent area, the Stono River estuary, approximately 20 km southwest of Charleston Harbor. This area is characterized as a well-mixed, C-type salt marsh estuary, with little freshwater inflow (Day et al., 1989). It has small tidal inlets with extensive mud banks and marsh vegetation and is influenced primarily by residential development. Dolphins using the CHS site are believed to show less evidence of compromised health than IRL dolphins. However, an increase in commercial development and the proximity of heavy industrial and agricultural influences in the CHS area suggest a potential for detrimental effects on dolphin health.

Serologic tests

Direct and modified agglutination tests: For direct agglutination test (DAT) mouse-derived whole formalin–preserved *T. gondii*, tachyzoites were used as the antigen, and sera were diluted in a buffer with 2-mercaptoethanol to remove nonspecific, naturally occurring immunoglobulin M (IgM)-like antibodies (Desmonts and Remington, 1980). The DAT was standardized for human sera at the Institut de Puériculture, Paris, France, and compared with the Sabin–Feldman dye test (DT) (Desmonts and Remington, 1980). The DAT measures only immunoglobulin G (IgG) antibodies. The DAT, modified slightly by Dubey and Desmonts (1987) and called the MAT, had mercaptoethanol added during the last step to reduce human exposure to the toxic fumes of mercaptoethanol. The antigen for both DAT and MAT is identical and is prepared at the Institut of Puériculture; this antigen is stable at 4 C for 6 mo or more. The DAT was performed at the Institut de Puériculture as described (Desmonts and Remington, 1980), and the MAT was performed at the APDL as described (Dubey and Desmonts, 1987). Sera were diluted 2-fold, starting at a 1:20 or 1:25 dilution.

Sabin–Feldman DT: Sera were diluted 2-fold, starting at a 1:10 dilution, and the test was performed as described (Desmonts and Remington, 1980).

Indirect fluorescent antibody test: The indirect fluorescent antibody test (IFAT) was performed at the APDL using a fluorescein isothiocyanate–conjugated anti-bottlenose dolphin IgG (heavy and light chain) rabbit conjugate (Bethyl Laboratories, Montgomery, Texas), which was diluted 1:25 before use. Sera were diluted 2-fold from 1:20 to 1:320.

Indirect hemagglutination test: The indirect hemagglutination test (IHAT) was performed by a commercial laboratory using the commercial test kit TPM-Test™ (Wampole Laboratories, Cranbury, New Jersey). Samples were screened at a dilution of 1:64 and then serially diluted 2-fold if positive.

Enzyme-linked immunosorbent assay: The enzyme-linked immunosorbent assay (ELISA) was performed per the manufacturer's instructions using a commercially available kit in a 96-well plate format containing a recombinant *T. gondii* P30 antigen (SafePath Laboratories, Carlsbad, California). The primary antibody from the dolphins was diluted 1:50, added to each well in duplicate, and incubated for 30 min. A 1:500 dilution of rabbit anti-bottlenose dolphin IgG-h+l horseradish peroxidase (HRP) conjugate (Bethyl) was used as the second-step antibody and was incubated for 30 min before color development using the chromogen tetramethybenzidine along with citric acid and peroxide. The reaction was stopped with 1 M phosphoric acid, and the reaction was read in an ELISA reader at 450/650 (Molecular Devices *V*max, Sunnyvale, California). Sera from captive-bred dolphins were used as a negative control. The ELISA values from the negative controls were subtracted from the ELISA values of the experimental dolphin sera; any experimental sera whose values exceeded 2 times the negative control value after the subtraction were reported as positive.

Western blot: Toxoplasma gondii tachyzoite proteins (200 μg) solubilized in Laemmeli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)–reducing sample buffer were electrophoresed on 4–12% gradient Bis-Tris (Bis-(2-hydroxyethyl)-amino-Tris(hydroxymethyl)-aminomethane) gels (Invitrogen, Carlsbad, California) in 50 mM MOPS-SDS running buffer (50 mM 3-N-morpholino propane sulfonic acid, 50 mM Tris base, 3.5 mM SDS, and 1 mM EDTA, pH 7.7) at 150 V for 75 min. Electroblotting was carried out on unfixed gels by the transfer of proteins onto Immobilon polyvinylidine difluoride nylon blotting membrane (Millipore, Bedford, Massachusetts) using a Novex gel transfer apparatus (Novex, San Diego, California) set at 40 V for 80 min in 25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, and 20% methanol, pH 7.2, blotting buffer. For Western blotting, the membranes were rinsed in TBS (a 50 mM Tris-buffered, 0.85% NaCl solution, and any unbound sites on the membrane were saturated with Detector Block solution (Kirkegaard and Perry, Gaithersburg, Maryland). The membrane was cut into strips, and each strip was incubated in a 1:100 dilution of dolphin serum overnight at 4 C. HRP-conjugated rabbit anti-bottlenose dolphin IgG-h+l as described above was used as the second-step antibody at a dilution of 1:800. The tachyzoite protein bands recognized by dolphin sera were visualized using the 4 CN membrane developer kit (Kirkegaard and Perry). Western blot images were captured using a Nikon digital imaging system.

RESULTS

2004 samples

All sera were positive by the MAT, and results were confirmed by the DAT (Table I). There was good agreement between MAT and DAT; titers were within 2–4 dilutions. By the DT, 7 dolphins had antibodies with titers of 1:800 or higher; all of these samples had comparable titers by the DAT and MAT. By the IHAT, 8 dolphins had antibodies (Table I). Of special interest are the IHAT-negative results from 2 dolphins (nos. 849 and 884), which had relatively high DT and MAT titers. The

TABLE I. Prevalence of *Toxoplasma gondii* antibodies in sera collected from dolphins in 2004.*

				Toxoplasma gondii titer						
ID	Sex	Capture location	Age (yr)†	MAT	DAT	IFAT	DT	IHAT	ELISA§	Western blot‖
800	M	SC	6	160	100	80	<10	<64	+	+[a]−[b]
814	M	SC	13	80	40	40	<10	<64	−	+/−
822	M	SC	4.5	40	20	20	<10	<64	−	+/−
831	F	SC	4.75	80	100	40	<10	<64	+	+/−
833	F	SC	5	160	200	20	<10	<64	−	+/−
845	F	SC	5	80	80	20	<10	<64	−	+/−
847	F	SC	3.5	40	20	<20	<10	<64	−	+/−
848	M	SC	ND‡	80	40	40	<10	<64	++	+/−
849	F	SC	5.5	>5,120	16,000	>320	>3,200	<64	+++	+/+
851	F	SC	3.5	80	40	40	<10	<64	+	+/−
853	F	SC	3.75	80	40	40	<10	<64	−	+/−
855	F	SC	ND	80	40	80	<10	<64	+	+/−
857	F	SC	ND	40	20	<20	<10	<64	−	+/−
874	M	SC	11	80	40	<20	<10	<64	−	+/−
876	M	SC	13	80	80	40	<10	512	+	+/−
878	M	SC	16	80	20	40	<10	<64	−	+/−
880	M	SC	16	1,280	4,000	>320	800	2,048	+++	+/+
882	M	SC	ND	40	40	20	<10	<64	+	+/−
884	M	SC	ND	>5,120	16,000	>320	>3,200	<64	+++	+/+
886	M	SC	21	80	40	160	<10	<64	++	+/−
888	M	SC	ND	80	40	40	<10	<64	+	+/−
890	M	SC	8	80	40	20	<10	<64	+	+/−
892	M	SC	24	80	40	40	<10	<64	+	+/−
894	M	SC	28	1,280	4,000	>320	1,600	2,048	+++	+/+
915	F	FL	7	40	20	40	<10	<64	+	+/−
929	F	FL	9.5	40	40	40	<10	<64	−	+/−
931	F	FL	ND	40	20	40	<10	<64	++	+/−
933	F	FL	4.5	40	40	40	<10	<64	−	+/−
934	M	FL	15	40	40	20	<10	<64	++	+/−
935	F	FL	10	40	100	20	<10	512	+	+/−
936	M	FL	15	80	100	40	<10	<64	+	+/−
937	F	FL	16	80	100	40	<10	<64	++	+/−
939	F	FL	3.5	80	40	40	<10	<64	++	+/−
941	F	FL	12.5	80	100	40	<10	<64	++	+/−
943	F	FL	12	40	40	40	<10	<64	++	+/−
945	F	FL	11	80	80	40	<10	<64	++	+/−
947	F	FL	10	40	40	40	<10	<64	−	+/−
949	F	FL	6	80	80	40	<10	<64	+	+/−
950	M	FL	27	40	80	40	<10	<64	+	+/−
951	F	FL	ND	40	80	40	<10	<64	+	+/−
954	M	FL	11	40	80	20	<10	<64	−	+/−
955	F	FL	ND	20	20	20	<10	<64	+	+/−
956	M	FL	13	80	40	40	<10	<64	+	+/−
957	F	FL	13	1,280	2,000	>320	800	2,048	+++	+/+
958	M	FL	9	80	100	40	<10	<64	−	+/−
960	M	FL	ND	2,560	2,000	>320	800	2,048	++++	+/+

TABLE I. Continued.

ID	Sex	Capture location	Age (yr)†	*Toxoplasma gondii* titer						
				MAT	DAT	IFAT	DT	IHAT	ELISA§	Western blot‖
961	F	FL	ND	80	40	40	<10	<64	+	+/−
962	M	FL	16	80	100	40	<10	<64	+	+/−
964	M	FL	8	80	80	40	<10	<64	+	+/−
966	M	FL	6	20	40	40	<10	<64	+	+/−
968	M	FL	11	80	200	40	<10	<64	+	+/−
970	M	FL	15	2,560	4,000	>320	1,600	2,048	+++	+/+
972	M	FL	5	40	100	20	<10	<64	−	+/−
974	M	FL	4.5	40	200	40	<10	<64	−	+/−
976	M	FL	8	40	40	40	<10	<64	+	+/−
978	M	FL	12	80	80	40	<10	<64	++	+/−
980	M	FL	9	20	20	40	<10	128	+	+/−
982	M	FL	11	40	20	20	<10	<64	+	+/−
984	M	FL	19	80	200	40	<10	<64	+	+/−
A0604	M	FL	18	80	40	40	<10	<64	+	+/−

* SC, South Carolina; FL, Florida; ID, identification number; MAT, modified agglutination test; DAT, direct agglutination test; IFAT, indirect fluorescent antibody test; DT, dye test; IHAT, indirect hemagglutination test; ELISA, enzyme-linked immunosorbent assay.

† Age denotes the individual's age in the year when the sample was taken.

‡ No data.

§ Corrected ELISA values; −, <0.5 ELISA units (EU); +, >0.5 EU; ++, 1.0–1.99 EU; +++, 2.0–2.99 EU; ++++, >3.0 EU.

‖ [a] Indicates recognition of bands other than the P30 in the Western blot; [b] indicates recognition of the P30 band and the other bands in the Western blot.

IFAT titers were <1:20 in 3, 1:20 in 11, 1:40 in 36, 1:80 in 2, 1:160 in 1, and 1:320 in 7.

The ELISA results closely followed the MAT results; all MAT values higher than 40 were identified as positive or strongly positive in the ELISA against the P30 antigen. Twenty-two MAT samples had values of 40 or less; of these, the ELISA identified 13 as positive, including 3 with values of 20 in the MAT. Western blots demonstrated recognition of multiple bands by each of the dolphin sera, but recognition of the P30 antigen, which is generally considered diagnostic for *T. gondii* exposure, was observed only with sera identified as 3+ or 4+ in the ELISA assay (Fig. 3).

2003 samples

All 86 dolphins had MAT antibodies with titers of 1:25 in 29, 1:50 in 23, 1:100 in 27, 1:200 in 3, 1:1,600 in 1, and 1:3,200 in 3; these sera were not tested by other means.

Repeat samples

Six dolphins were caught in both 2003 and 2004; their MAT titers were similar.

DISCUSSION

Results of the present study confirm earlier observations (Dubey et al., 2003) that most free-ranging dolphins examined from U.S. coastal waters have *T. gondii* antibodies detected by the MAT. At present, there are no corresponding parasite recovery data to confirm the validity of the MAT for dolphin sera. In contrast, the MAT has been extensively validated by detecting *T. gondii* parasites in tissues from naturally infected pigs (Dubey, Thulliez et al., 1995; Dubey, 1997) and chickens (Dubey, Marcet, and Lehmann, 2005, and references therein) whose sera were MAT positive. If there are factors in dolphin sera that reacted nonspecifically with *T. gondii* in the DAT in the present study, these substances were not present in the sera of dolphins from the Spanish Mediterranean coast, where MAT antibodies were found in only 4 of 36 striped dolphins (*Stenella coeruleoalba*) and 4 of 7 bottlenose dolphins (Cabezón et al., 2004).

Murata et al. (2004) tested 59 sera from 40 bottlenose dolphins by the IHAT and the latex agglutination test (LAT); these dolphins were kept in aquaria in Japan for 2–22 yr after they had been captured from the sea. Antibodies to *T. gondii* were found by both the IHAT (with dilutions of 1:160 or higher) and the LAT (with dilutions of 1:16 or higher) in 6 dolphins. Determination of immunoglobulin type suggested that one of the dolphins acquired the infection in captivity.

The Sabin–Feldman DT is considered the most specific test for toxoplasmosis in humans. In the present study, 7 of 60 dolphins were positive with the DT. However, the DT is a complement-based test and is known not to work with chicken sera (Dubey et al., 1993, and references therein) or cattle sera (Dubey et al., 1985).

The ELISA test in the present study indicated exposure to *T. gondii* in 44 of 60 dolphins; control sera from captive-bred dolphins with no history of exposure to coastal waters were used to validate the positive test results. The recombinant antigen used in the commercial test, the major surface antigen P30 (SAG 1) of *T. gondii*, has been used extensively in diag-

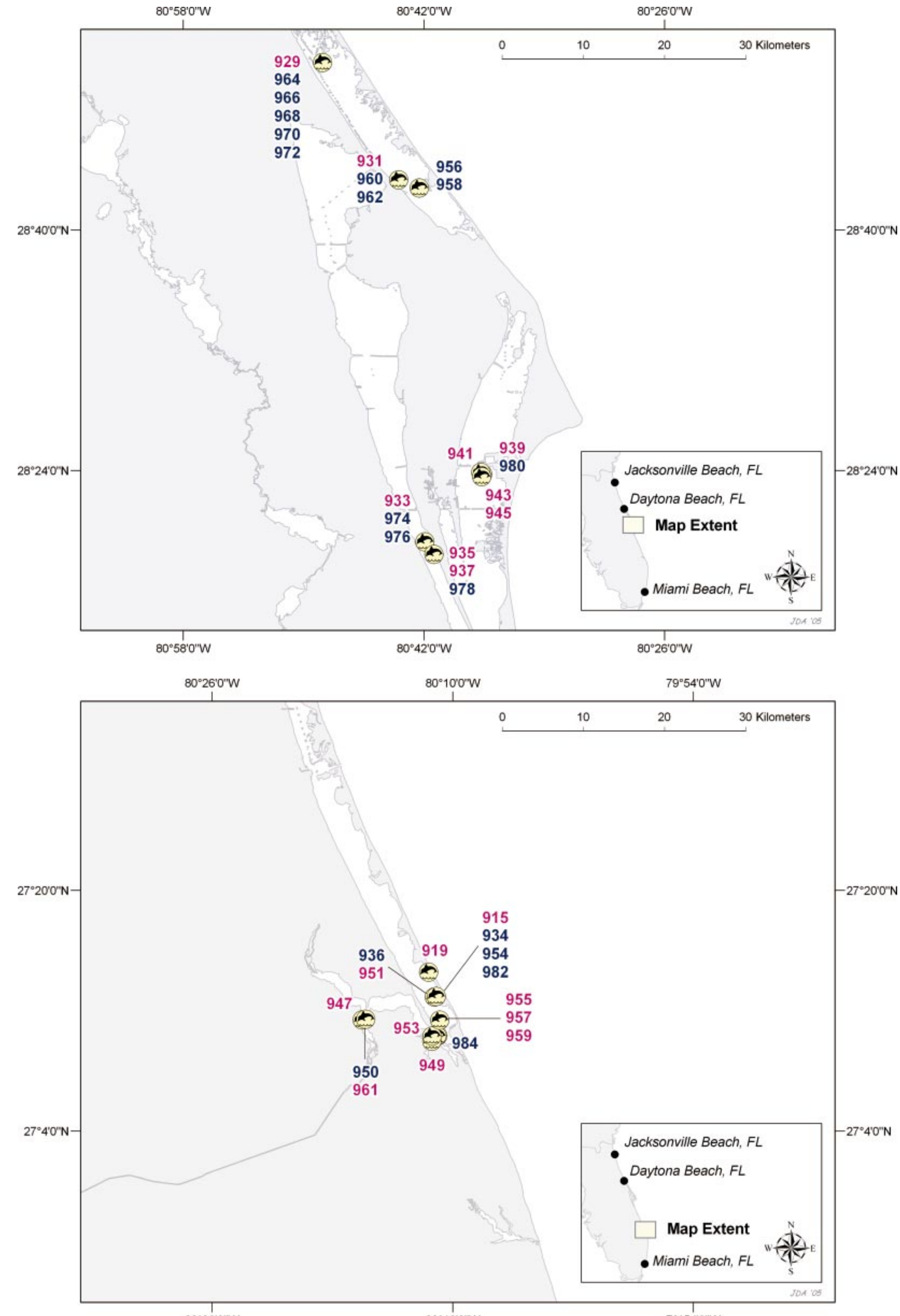

FIGURE 1. Maps of the northern (top) and southern (bottom) Indian River Lagoon, Florida, study area showing locations of dolphins tested in 2004 for *Toxoplasma gondii* (red = females; blue = males).

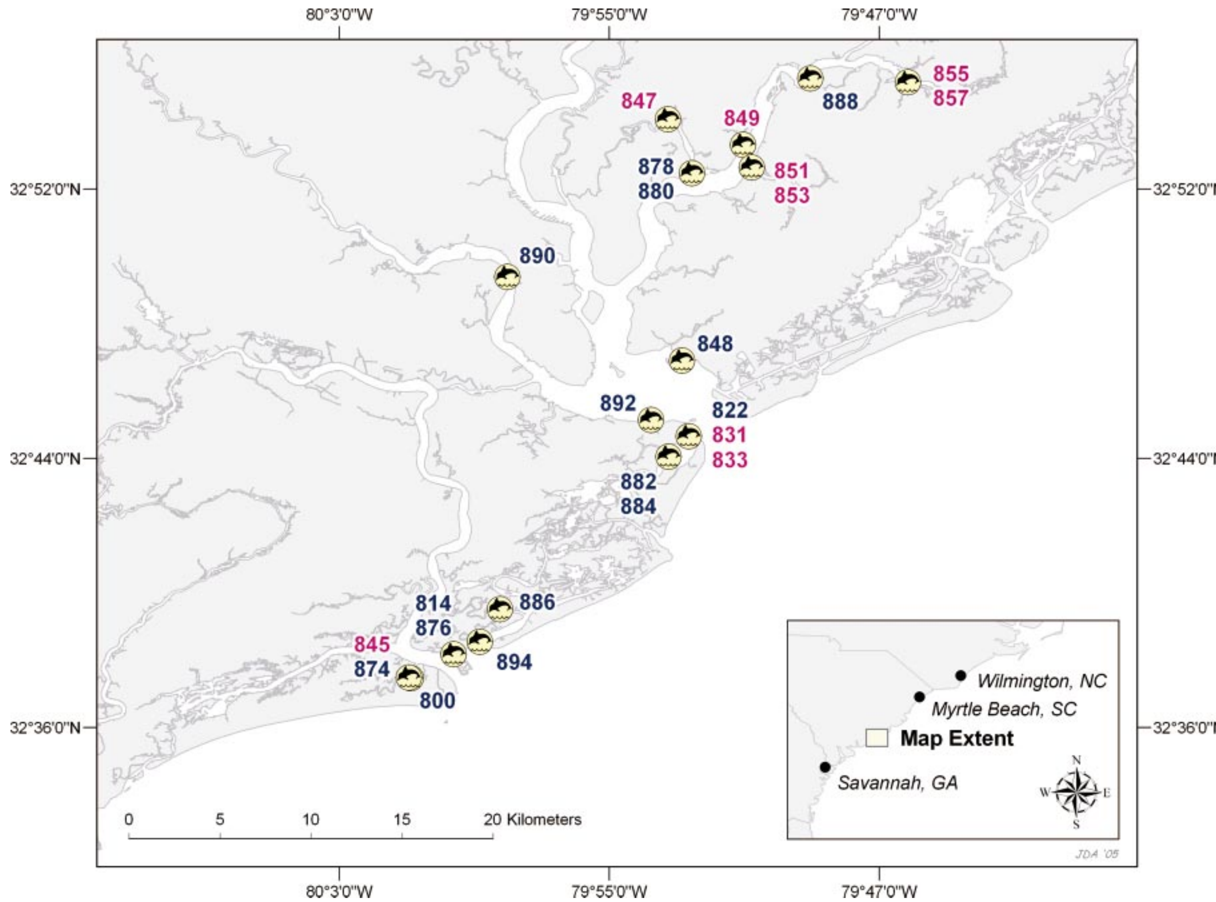

FIGURE 2. Map of the Charleston, South Carolina, study area showing locations of dolphins tested for *Toxoplasma gondii* in 2004 (red = females; blue = males).

nostic tests for humans and animals (Aubert et al., 2000; Sager et al., 2003).

Western blot results confirmed the recognition of the P30 antigen by the dolphin sera that were scored as 3+ or 4+ in the ELISA and the lack of reactivity to the antigen of sera from animals with scores of 2+ or less in the ELISA and the control sera from captive-bred dolphins. Protein bands other than the P30 antigen were recognized by all of the experimental dolphin sera, but not by the sera from the captive-bred dolphins. Recognition of the P30 antigen by the high-titered sera may indicate the increased avidity of these sera to the P30 antigen in animals that had been infected for a longer time period, as was seen with sheep (Sager et al., 2003).

In the present study, antibodies to *T. gondii* were detected in only 8 of 60 dolphins by the IHAT; however, in several earlier studies, the IHAT was very insensitive (Dubey and Beattie, 1988; Dubey and Thulliez, 1989; Dubey, Lappin, and Thulliez, 1995; Dubey, Thulliez et al., 1995), a response repeated in the present study and found to differ from the 3 other tests that were used.

Locations from which dolphins were sampled for *T. gondii* serological testing during 2004 in the IRL are shown Figure 1A, B and, for the CHS site, such locations are shown in Figure 2. The same capture sites were also used for the collection of samples during 2003 (data not shown). Since all dolphin samples tested positive to *T. gondii* throughout the range of both capture sites, there appear to be no areas within the sites in which animals were not seropositive. Animals with higher titers as tested by the MAT were not found in any particular area; there did not appear to be gender- or age-related patterns.

How marine mammals become infected with *T. gondii* is unknown. Ingesting oocysts directly from the water and ingesting tissues of animals that are infected with *T. gondii* and harbor infective parasites in their tissues are the most likely explanations. Felids are the only known hosts that excrete environmentally resistant oocysts. Individual cats can excrete millions of oocysts, and there are more than 100 million cats in the United States (Dubey, 2004). Miller, Gardiner, Kreuder et al. (2002) presented evidence that coastal freshwater surface runoff presented a risk of infection to sea otters, so it is possible that *T. gondii* oocysts could be washed into coastal waters via runoff contaminated by cat excrement.

The role of marine invertebrates in the life cycle of *T. gondii* is unknown. Although *T. gondii* does not parasitize cold-blooded animals, molluscs are filter feeders and may thus concentrate oocysts. *Toxoplasma gondii* infection of dolphins is even more intriguing because dolphins drink little or no water. Their water requirements are satisfied by the fish, squid, or other cold-blooded sea animals they consume. Finding *T. gondii* antibodies in 100% of the wild Atlantic bottlenose dolphins from the United States raises 2 concerns. First, the specificity of low antibody titers needs to be confirmed by detecting *T. gondii* in dolphin

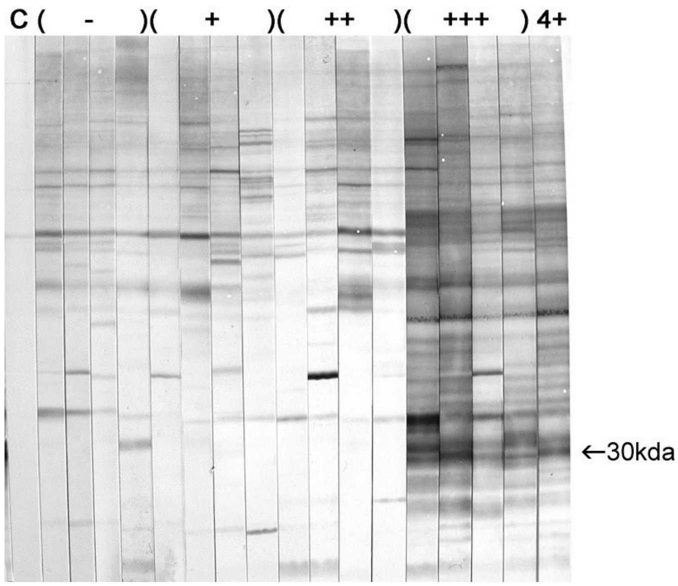

FIGURE 3. Western blots of *Toxoplasma gondii* tachyzoite extract electrophoresed on 4–12% gradient Bis-Tris (Bis-(2-hydroxyethyl)-amino-Tris(hydroxymethyl)-aminomethane) gels. Blotted protein exposed to bottlenose dolphin sera diluted 1:100, followed by horseradish peroxidase (HRP)-conjugated rabbit anti-bottlenose dolphin sera diluted 1:800. The *T. gondii* P30 antigen was specifically recognized by dolphin sera with scores of 3+ or 4+ in the enzyme-linked immunosorbent assay (ELISA) (arrow). Numerous protein bands were recognized in other experimental sera but not with control sera from captive-bred dolphins. Four representative strips from each ELISA scoring category shown in order (control = C1 sera = 814, 878, 954, 974; 1+ sera = 876, 956, 961, 984; 2+ sera = 848, 886, 941, 978; 3+ sera = 849, 884, 894, 957; and the single 4+ sera = 960).

tissues histologically or by bioassay. Second, if the serologic data from the present study indicating the high prevalence of infection in dolphins are confirmed by such testing, the extent of environmental contamination with *T. gondii* that gives rise to such a high prevalence in dolphins might also present a risk to human health.

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